A method of analyzing procoagulant activity in monocytes at single cell level

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Background and Objectives. Procoagulant activity (PCA) of monocytes is known to play a pivotal role in a variety of physiologic and pathophysiologic processes, such as disseminated intravascular coagulation, atherosclerosis, arterial and venous thromboembolism, cancer-related hypercoagulability and immunopathologies. Until now, PCA has been studied by clotting assays of a whole cell population or at single cell level by analyzing tissue factor antigen, the protein that initiates PCA but does not always correlate with it. Here, we describe a new simple flow cytometric method that allows the PCA of monocytes to be studied at a single cell level by quantifying the fibrin formed around the cells in suspension.

Design and Methods. Purified fibrinogen was tagged with FITC and added to a recalcified *developer plasma* containing suitable amounts of heparin in order to inhibit the expansion of clotting, thus limiting the formation of fibrin to the surface of cells with PCA. With appropiate amounts of heparin, in 10 min, large *sea urchin*-like cells with fibrin needles around some monocytes were formed and, after fixation, cytofluorimetrically analyzed.

Results. Blood mononuclear cells isolated and immediately analyzed showed less than 0.1% *sea urchin* cells. Adherence alone, lipopolysaccharides or ionomycin stimulated expression of PCA in a dose- and time-dependent relationship: after 30 min, 1-3% of the MNC showed PCA, and after 20h this reached 5-10%. Density separation of monocytes showed that different stimulators act on different maturation stages. Subjects with diabetes express more monocytes with PCA than normal subjects after 30 min stimulation.

Interpretation and Conclusions. This method allows PCA analysis of monocytes at single cell level and requires only a low number of cells. The signal produced by the fluorescent fibrin is strong and easily analyzed by flow cytometry. The method is suitable for analyzing blood from patients with different pathologies and many conditions under different stimuli.

Key words: monocytes, tissue factor, procoagulant activity, flow cytometry.

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The procoagulant activity (PCA) of monocytes is known to play a pivotal role in a variety of physiologic and pathophysiologic processes, such as disseminated intravascular coagulation, atherosclerosis, arterial and venous thromboembolism, cancer-related hypercoagulability and immunopathologies.[1] Tissue factor (TF) is the primary cellular initiator of the PCA that is expressed by activated monocytes and activated endothelial cells. TF is a 47 kDa membrane-bound glycoprotein that initiates blood coagulation after interaction with factor VIIa (the extrinsic clotting pathway) resulting in a complex that acquires catalytic activity. The primary substrates for the TF-FVIIa complex are FIX and FX, which are converted to their active derivatives FIXa and FXa, respectively, thus leading to thrombin generation, which in turn cleaves fibrinogen to fibrin.[2] Peripheral blood monocytes have been studied after *in vitro* culture with stimulators such as lipopolysaccharide (LPS), mitogens, and Ca^{++} ionophores. Generally, the TF expressed by these cells has been quantified as total PCA activity of intact or disrupted cells by analyzing the capacity to accelerate the coagulation of recalcified plasma. Recently, using a monoclonal antibody specific for TF and cytofluorimetry, it has become possible to analyze TF antigen at the level of individual cells. However, TF antigen does not always correlate with PCA because others molecules, such as tissue factor protein inhibitor (TFPI), or the phospholipid composition of the membrane, can modulate the activity of the prothrombinase complex.[3,4] Here, we describe a new, simple, flow cytometric method that allows the PCA of single monocytes to be studied by quantifying the fluorescent fibrin formed around the cells in suspension.

Design and Methods

Lyophilized human fibrinogen was obtained from Kabi (Stockholm, Sweden). Fluorescein isothiocyanate (FITC), dialysis tubes, *E. coli* LPS, ionomycin, thromboplastin, human plasma deficient in factors X, VIII, and VII, and Histopaque were all obtained from Sigma Chemical Co. FITC- and phycoerythin (PE)-tagged isotype-matched irrelevant control monoclonal antibodies IgG1 and IgG2a, and anti CD14-PE were obtained from Pharmingen (San Diego, CA, USA) and anti TF-FITC was supplied by American Diagnostica Inc. (Greenwich, CT, USA).

Isolation of cells

Mononuclear cells (MNC) were obtained from whole blood collected from healthy donors and anticoagulated with 0.1 vol of 3.8% sodium citrate. One volume of blood was layered over one volume of Histopaque and cen-

trifuged at 400g for 10 min. The MNC layer was aspirated and washed twice with citrate-saline (0.15 mol/L NaCl containing 10 mmol/L sodium citrate) at 100 g for 5 min to minimize contamination by platelets. The cells were then resuspended at 1×10^6/mL in RPMI containing 10% human serum as complete medium (CM).

Isolation of MNC with different densities

After the MNC had been obtained and washed as decribed above, the sedimented cells were resuspended in 1 mL of saline + 10 mM Hepes buffer and layered on a cushion made up of multiple layers of Histopaque previously prepared as follows: 50 µL of a 5% solution of human albumin (for intravenous use) were added to 4 mL of Histopaque (HP) in a 15 mL tube, mixed trying to wet the internal wall uniformly. The proteins adsorbed onto the walls facilitate the construction of the layers. The Histopaque with protein was transferred to a second tube and the first one was used to deposit the denser layer made of 0.5 mL of 100% HP. Another layer of 0.5 mL of HP, this time containing 2.5% saline, was formed by carefully sliding it down the internal wall over the first layer. This was followed by a layer of 0.5 mL HP containing 5% saline and, in succession, by other layers of 0.5 mL HP containing 7.5%, 10%, 12.5%, 15% and 17.5% saline. This series of HP solutions was made by adding small fractions of saline to the second tube with HP until the desired concentration was obtained and withdrawing 0.5 mL to construct the gradient on the first tube.

After centrifugation for 10 min at 400 g the MNC located in the 5 fractions, of visibly different densities against a back light, were aspirated into tubes that had been wetted internally with a 5% solution of human albumin, washed twice with saline containing 10 mmol/L sodium citrate and resuspended in CM. Generally, the lower density MNC fraction contained 30-60% monocytes, while the higher density fractions contained 2-8% monocytes.

Isolation of monocytes

In order to separate the monocytes, 60 mm Petri dishes were incubated with 10% citrated human plasma in PBS for 5 min and then washed with PBS. MNC resuspended in CM at 2×10^6/mL were added and after 60 min adherence at 37°C in 8% CO_2, the non-adherent lymphocytes were removed.

The adherent monocytes were recovered after incubation with saline containing 10 mmol/L sodium citrate for 20 min at 4°C, released after repeated pipetting and after centrifugation, the sedimented cells were resuspended in CM.

Determination of total monocytic cell surface TF activity

Total PCA on the surface of intact monocytes was determined by a single-stage clotting assay.[5] Briefly, 50 µL of mononuclear cell suspension were mixed with 50 µL of 25 mmol/L $CaCl_2$. Clotting was initiated by the addition of 50 µL of normal human plasma, and the clotting time at 37°C was recorded using a KC10 coagulometer (Heinrich Amelung, GmbH, Lemgo, Germany). The PCA was extrapolated from a standard curve drawn using a rabbit brain thromboplastin standard (Ortho, Raritan, NJ, USA). The cell surface-associated PCA was linear over the range of 12×10^6 mononuclear cells/mL. The PCA of monocytes was confirmed to depend on TF, because it was inhibited by factor VII-deficient plasma.[5]

Preparation of FITC-labeled fibrinogen

We used the method described by Faraday *et al.*[6] to prepare the labeled fibrinogen. Kabi fibrinogen was dissolved in saline at 10 mg/mL and dialyzed against 0.01 mol/L sodium borate + 0.15 mol/L sodium chloride buffer (pH 9.5) overnight. Next, 10 to 12 mg/mL fibrinogen were incubated with FITC (2 µg/mg fibrinogen) for 2 to 4 hours at 22°C. The excess FITC was removed by exhaustive dialysis against 0.1 mol/L Tris-HCl, pH 7.5, and phosphate-buffered saline solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L $Na_2HPO_4.7H_2O$, and 1.4 mmol/L KH_2PO_4). Each FITC-labeled fibrinogen preparation was stored at 4°C and used within 3 months without any decreased activity over that period.

Activation of monocytes (as MNC or a purified population)

LPS (1 mg/mL in ethanol) or ionomycin (1 mg/mL in ethanol) was diluted to 1/100 in CM and appropriate quantities were added to cells in CM or heparinized blood to obtain the desired concentration. The cells were incubated at 37°C in 8% CO_2 for variable periods of time.

Recovery of cells

After incubation, with or without stimulation, in Petri dishes, the MNC or purified monocytes were recovered by adding sodium citrate at a final concentration of 10 mmol/L and by incubating at 4°C for 10 min. After pipetting, the resuspended cells were washed once with saline containing 10 mmol/L Hepes + 10 mmol/L sodium citrate. The sedimented cells were then analyzed for their PCA or TF antigen content. In some experiments, whole blood stimulated with LPS for different times was layered on Histopaque, centrifuged, the MNC fraction was washed with saline containing 10 mmol/L Hepes + 10 mmol/L sodium citrate and the sedimented cells were analyzed as indicated above. In others experiments, stimulated and washed cells were fixed by adding an identical volume of saline containing 0.4% formaldehyde for 10 min and then washed once with saline.

Demonstration of cells with PCA forming fibrin-FITC stars or sea urchin cells

Simply adding activated monocytes with PCA to recalcified plasma produced a diffuse coagulation within 1-2 minutes that did not permit easy handling of the cells. However, the addition of heparin was effective in slowing down the entire process and permitted the fixation of monocytes during the generation of polymerized fibrin due to the high concentration of PCA on the membrane. After several experiments we observed that delaying the formation of fibrin around the cells by 10 minutes resulted in more dense fibrils.

The *developer* plasma was prepared immediately prior to use. First, a pool of citrated plasma obtained from 10 normal donors was prepared (from tubes typically used for coagulation analyses: 9 parts of plasma and 1 part of 3.7% sodium citrate solution). To 1 mL of this plasma, the following were added: i) 20 µL of 1 mol/L solution of calcium chloride, ii) 10 µL of the fibrinogen-FITC solution, iii) 10 µL of saline containing 1% to 5% low molecular weight (LMW) heparin (Seleparin-Italfarmaco for i.v. use) depending on whether the cells had low or high PCA, respectively (initially, 10 µL of saline containing 50 to 200 U of unfractionated heparin/mL were used). In some experiments, plasma deficient in factor X, VIII or VII was used instead of normal plasma.

Two hundred microliters of the *developer* recalcified plasma containing fibrinogen-FITC and heparin were distributed into a small tube and then 10,000-30,000 MNC or monocytes in 10 mL were added, mixed and incubated at room temperature without vibration for 5-10 min to permit the formation of the first fibrin needles. During this period, microscopic observation of 10 µL volumes on slides determined the time necessary for optimal development of the polymerized fibrin irradiating from the membrane of some monocytes (these elements appeared as *fibrin star* cells or *sea-urchin*-like cells). Once the *sea urchin* cells were clearly visible and well developed, the contents of the small tubes were fixed by adding 500 µL of saline containing 2% formaldehyde. Microscopic observation was no longer necessary when the best time had been determined for each particular combination of cells and stimuli. Flow cytometric analysis was performed within 1 h.

Separation of high molecular weight heparin

By gel filtration, 500 µL of crude heparin were sieved on a 10 cm column filled with 10 mL of G-25 Sephadex in saline and the void volume of 1 mL containing high molecular weight heparin was collected.

Flow cytometry analysis of PCA on monocytes

Suspensions of fixed cells without fluorescence and fluorescent fixed *sea urchin* cells were analyzed with a flow cytometer (Cytoron, Ortho). All parameters, fluorescence and light scatters, were acquired in the logarithmic mode. Data were acquired from 5,000 to 15,000 cells. The right and forward scatter diagram identified lymphocytes, monocytes without PCA (that had greater side scatter than lymphocytes) and monocytes expressing PCA, which had the greatest side scatter due to fibrin needles covering the cells. Green fluorescence of high intensity was derived only from those cells with the greatest side scatter and these two parameters were strongly correlated. Analysis was performed using a cytogram with green fluorescence as the Y axis and side scatter as the X axis. Some experiments were done to confirm that the population with *high side scatter-high green fluorescence* corresponded to the *sea-urchin* cells. Procedures to deplete and to enrich these populations were performed: diluting the fixed cells with 4 volumes of saline in a tube and layering over two volumes of normal citrated plasma produced, in 30 min at 1 g, a distinct prevalent sedimentation of *sea-urchin* cells in the bottom layer and a relative depletion of these cells in the upper layer, confirmed by fluoresence microscopy. In another experiment, developer plasma from which the FITC-fibrinogen had been omitted produced a population of high side scatter *sea-urchin* without green fluorescent cells.

Flow cytometry analysis of TF antigen

Cell surface expression of TF antigen was assessed by direct immunofluorescence. Cells were incubated (30 min, 4°C) with FITC-tagged monoclonal antibody against TF and with PE-tagged monoclonal antibody anti CD14. FITC- and PE-tagged isotype-matched irrelevant control antibodies served as the negative control for detecting non-specific fluorescence. Cells were analyzed after a single wash. Quadrant analysis of two-color dot-plot histograms was performed by setting the horizontal and vertical cursors on the isotypic negative control antibody to ensure that there were less than 0.5% positive cells.

Statistical analysis

Statistical analysis was performed with a two-tailed Student's t test. A value of $p<0.05$ was considered statistically significant.

Results

Different concentrations of heparin facilitate visualization of monocytes with different PCA contents

Recalcified citrated plasma without heparin mixed with monocytes containing PCA led to coagulation that initiated around the monocytes and rapidly spread through the whole plasma within minutes. Small amounts of heparin were added to slow down the process. Microscopic observations were used in

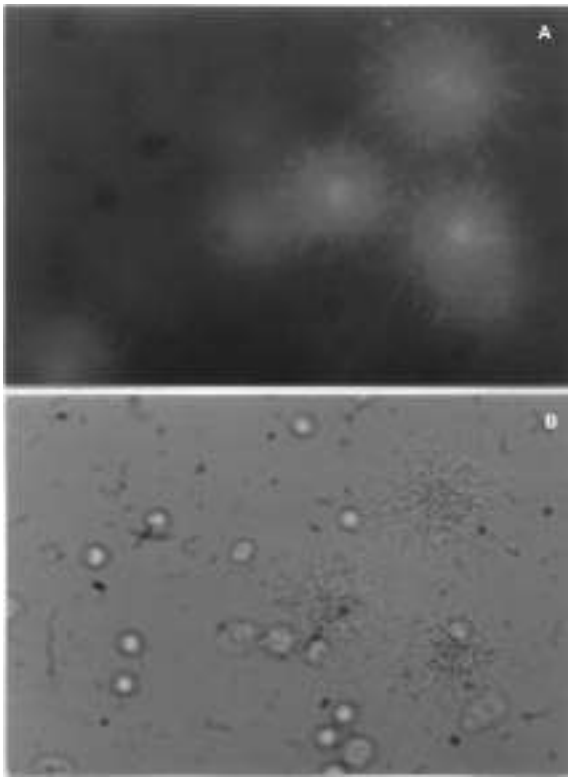

Figure 1A. Three *sea urchin* cells among several, small lymphocytes and two monocytes near the center of the microphotograph, without fibrin needles. This preparation of cells in suspension shows that the cells surrounded by fibrin do not reach the plane of the lymphocytes sedimented on the glass (seen slightly out of focus). Figure 1B: fibrinogen-FITC fluorescence localized on the needles of the same *sea urchin* cells shown in 1A.

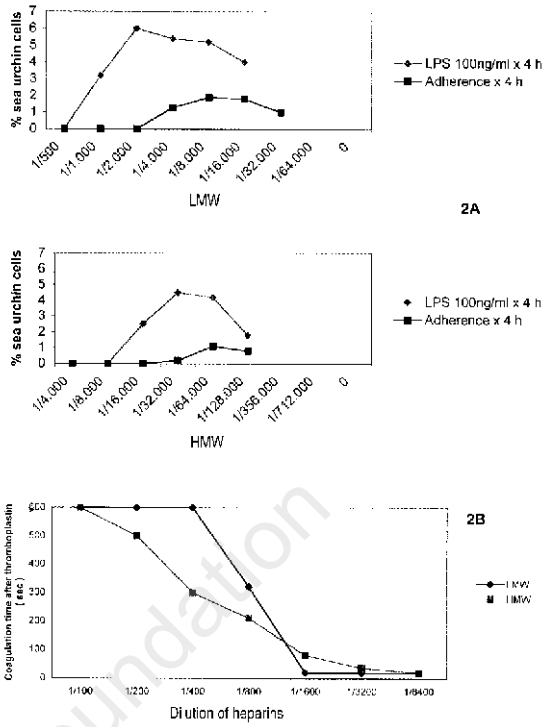

Figure 2. A. Percentage of monocytes expressing PCA visualized as *sea urchin* cells by different concentrations of LMW and HMW heparins and by two types of stimulation that generate different quantities of PCA: high by 100 ng/mL of LPS + adherence for 4 hours and low by simple adherence for 4 hours. Insufficient concentrations of heparins caused lower percentages of *sea urchin* cells because the cells agglutinated due to the excessive fibrin formed. B. Coagulation time obtained with different concentrations of LMW and HMW heparins added to recalcified plasma. A small amount of human thromboplastin started the process. LMW heparin showed a curve with a steep descent.

order to determine the amount of heparin needed in the recalcified plasma to permit the formation of well-shaped *sea urchin* fibrin around the monocytes with PCA in 10 min while at the same time avoiding widespread coagulation (Figure 1). Initially we found that monocytes containing a low level of PCA, such as that expressed spontaneously after 60 min adherence to plastic at 37°C, show better fibrin formation around the cells when a developer plasma containing 0.5 U/mL of unfractionated heparin was used, while those monocytes that expressed high PCA after 16 hours' stimulation with LPS needed 2 U/mL of unfractionated heparin to maintain the fibrin formation around the cell.

However, investigating the effect of heparins of different molecular weights, we found that a wider range of concentrations of low molecular weight (LMW) heparin than of unfractionated heparin or high moleular weight heparin could be added to the recalcified plasma to inhibit the diffuse coagulation while still allowing fibrin formation around cells with PCA (Figure 2A). The heparins also acted differently on the coagulation of recalcified plasma when thromboplastin was used as the initiator of the coagulation cascade: when added LMW heparin reached an anticoagulant concentration, it resulted in a sudden, fast prolongation of coagulation (steep curve in the graph) whereas adding high molecular weight heparin resulted in a slow prolongation of the coagulation time (shallow curve) (Figure 2B). Unfractionated heparin produced intermediate values (*results not shown*). After these findings, we used LMW heparin in the developer plasma at final concentrations of 1/10,000 and 1/2,000 with cells with low or high PCA, respectively. Figure 3 shows the typical cytograms with *sea urchin* cells from previously separated MNC or purified monocytes stimulated with LPS in complete medium and the

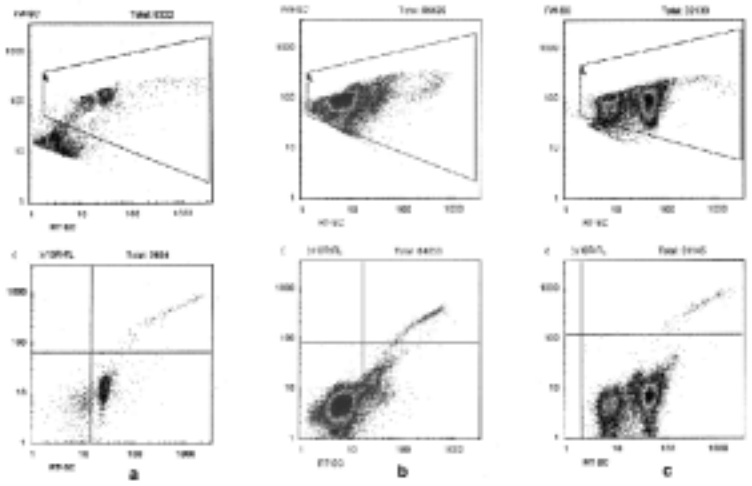

Figure 3. Cytograms obtained analyzing PCA on cells after stimulation of purified monocytes (a), MNC (b) and heparinized whole blood (c) with 100 ng/mL LPS for 4 h. The *sea urchin* cells showed the greatest value of right scatter (more than neutrophils) that could be best resolved against green fluorescence.

cytogram obtained after stimulation of whole heparinized blood with LPS followed by separation with density gradient (neutrophil contamination of MNC due to activation by LPS does not hamper the analysis of fluorescent *sea urchin* cells).

Time- and dose-dependency of PCA following stimulation

Table 1 summarizes the results obtained with different stimuli for different times on MNC incubated in complete medium. Mononuclear cells did not express PCA immediately after separation, but incubated at 37°C in 8% CO_2 with complete medium in Petri dishes, between 0.1 to 1% of monocytes expressed PCA after 60 min adherence. These mononuclear cells were demonstrated as *sea urchin* cells after 10 min incubation at room temperature with the developer plasma containing low amount of LMW heparin (1/10,000). Adherence alone, over time, increased the percentage of monocytes with PCA, which reached up to 5 - 10% after 16 hours. Furthermore, overnight stimulation with LPS increased the percentage of monocytes with PCA to 20 - 30% but the potency of PCA-bearing cells increased so much that these *sea urchin* cells were shown better by developer plasma containing a higher amount of LMW heparin (1/2,000). After adherence, addition of stimulators such as ionomycin or LPS markedly increased the percentage of monocytes with PCA (1-3% after 60 min, 10-30% after 16 h) and the quantity of fibrin per cell. When MNC were incubated in small diameter tubes with frequent agitation (conditions allowing minimal adherence), less than 0.2% of the monocytes expressed PCA spontaneously after 30 min but with LPS as a stimulator in the same conditions, 1% to 5% of monocytes clearly showed PCA. Remarkably, if LPS was added after 10 min adherence in Petri dishes, very little PCA was detected after 30 min (less than 0.2% of monocytes) but increased after that time. The lack of activity observed in complete medium containing EDTA indicates that extracellular calcium is necessary for the induction of PCA. MNC incubated with ionomycin in RPMI without serum expressed PCA but the number of monocytes recovered was low because of the increased cell loss in such conditions. The number of monocytes with PCA on the surface after stimulation increased in parallel with the serum concentration reaching a maximum at 10% and then declined. However, in all circumstances, the presence of serum in RPMI favored PCA expression. Using fetal calf serum (FCS) instead of human serum at the same concentration resulted in a slight enhancement of the number of monocytes expressing PCA and the amount of fibrin formed by each cell.

No *sea urchin* cells were formed when recalcified factor VII-deficient plasma or factor X-deficient plasma was used, whereas fibrin needles formed around activated monocytes with factor VIII-deficient plasma, suggesting that the procoagulant activity was attributable to membrane-bound active tissue factor, as observed previously.[8,9] We also tried to preserve cells with PCA to permit analysis for some days after different stimuli and found that light fixation with a final concentration of 0.2% formaldehyde in saline for 10 min provided the best combination for morphology and PCA studies after many days of storage at 4°C. This procedure allowed comparison of the intensity and the number of monocytes with PCA expressed at different times and in different conditions using only one batch of developer plasma.

Table 1. Percentage of MNC with PCA forming *sea urchin* cells obtained after different stimulations for different times.

Cells and type of stimulation	*% MNC with PCA*
Not stimulated, analyzed after separation	0.02
1 ng/mL LPS for 30 min	0.1
100 ng/mL LPS for 30 min	0.5
1 µg/mL LPS for 30 min	1.1
1 µg/mL LPS for 60 min	1.9
1 µg/mL LPS for 120 min	2.6
1 µg/mL LPS for 240 min	3.4
1 µg/mL LPS for 20 h	8.6
1 µg/mL Ionomicyn for 30 min	0.3
1 µg/mL Ionomicyn for 60 min	0.7
1 µg/mL Ionomicyn for 120 min	1.4
1 µg/mL Ionomicyn for 240 min	3.1
1 µg/mL Ionomicyn for 20 h	5.2
Adherence for 30 min	0.2
Adherence for 60 min	0.5
Adherence for 120 min	1.1
Adherence for 240 min	2.9
Adherence for 20 h	4.2
1 µg/mL LPS for 240 min with EDTA	0.1
1 µg/mL LPS for 240 min in RPMI+1% serum	1.5
1 µg/mL LPS for 240 min in RPMI+3% serum	2.5
1 µg/mL LPS for 240 min in RPMI+10% serum	3.8
1 µg/mL LPS for 240 min in RPMI+20% serum	3

Unless otherwise specified, MNC were cultured in 12-well plates at 1,000,000 cells/mL in RPMI plus 10% human serum and incubated at 37°C in 8% CO_2. Results shown are representative of three similar experiments.

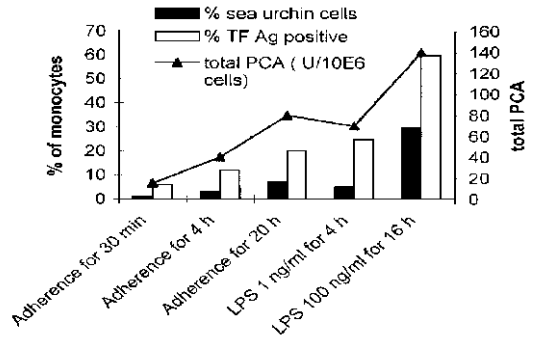

Figure 4. Relationship between the percentage of monocytes expressing TF antigen, percentage of monocytes with PCA forming *sea urchin* cells and the total PCA content of MNC obtained analyzing MNC obtained from one normal donor stimulated in different ways. Results shown are representative of two similar experiments.

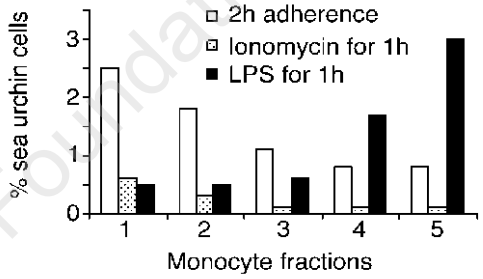

Figure 5. MNC obtained from five layers of density gradients were stimulated with adherence alone for 2 h, with ionomycin + adherence for 1h and with LPS without adherence for 1h. Because the presence of monocytes varied greatly among MNC in these layers, the percentage of "sea urchin" cells was calculated from the cells in the monocyte region, clearly distinct from the lymphocyte region in the cytograms.

Figure 4 shows the relationship between TF antigen expression on monocytes, the PCA results obtained with the method described here and the total PCA evaluated by the clotting assay on the same MNC stimulated in different ways. The close correlation between the results of these assays is obvious, but the percentage of monocytes with TF antigen was always superior to the percentage of monocytes with PCA detected by *sea urchin* cell formation: from 5-fold higher when a low dose of LPS or short time of adherence was used, to 2-fold higher when maximun expression of PCA was reached with a high dose of LPS or 20 h adherence.

Subpopulations of monocytes respond to stimulators differently

To investigate whether the monocytes easily forming PCA have a characteristic density, we separated five populations of monocytes according to their different densities. This procedure provided five layers of MNC in which CD14, the monocyte marker, showed five different mean intensities: 110, 116, 124, 133, 140 arbitrary units of fluorescence in a log scale and 108, 118, 130, 145, 152 arbitrary units of right scatter in a linear scale, respectively, from the first, upper, less dense to the fifth, lower, more dense layer. As shown in Figure 5a, the monocytes present in the lower density fraction developed more PCA than those with higher density after 2 hours' adherence (2.5%, 1.8%, 1.1%, 0.8%, 0.8% in cells fractions of increasing density). Ionomycin without adherence and after 60 min stimulated PCA poorly but nevertheless more strongly in the low density monocytes (0.6%, 0.3%, 0.1%, 0.1%, 0.1% respectively), stimulation for only 30 min did not produce demonstrable PCA.

However, LPS stimulation without adherence produced higher PCA in monocytes separated in the lower, more dense fraction. After 60 min with LPS without adherence, 3% of the more dense monocytes expressed PCA while only 0.5% of the less dense monocytes showed PCA. This observation is consistent with the idea that monocytes are a heterogeneous, differentiating population and that different stimuli can activate different stages of maturation.

Diabetic subjects have more monocytes expressing PCA than normal subjects

Advanced diabetes is a condition well-known to have high procoagulant status. In order to investigate whether this could be correlated to monocyte PCA expression, the MNC from 20 diabetics with high levels of glycosylated hemoglobin (range: 80 to 110 mg/L) and from blood donors with normal levels (range: 35 to 60 mg/L) were separated and stimulated with LPS without adherence (Figure 6). We found that after 30 min the percentages of monocytes expressing PCA in diabetic subjects were, despite the wide range of values, significantly higher than those found in normal donors (3.2% vs 1.4%, $p<0.05$). However, after 4 hours' stimulation, the percentage of monocytes expressing PCA was similarly high in both groups (6.5% vs 6%). It is noteworthy that the complete medium used during stimulation contained normal serum. This finding confirms Nijziel's published work on the plasma properties regulating TF activity on monocytes[7] and may indicate that the expression of PCA on the surface of monocytes in some pathologic condition is complex and requires further studies.

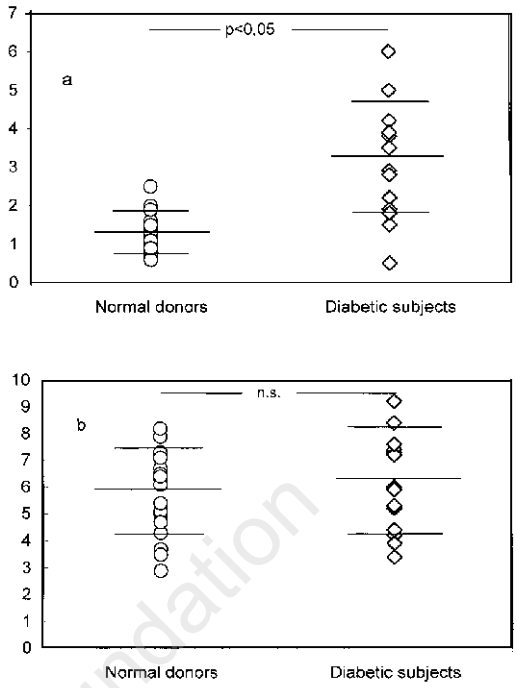

Figure 6. Diabetic subjects had a higher percentage of monocytes forming *sea urchin* cells than did normal blood donors after a brief stimulation. The MNC (1,000,000 MNC/mL of CM) were stimulated with 100 ng/mL of LPS for 30 min (a) and for 4 h (b) in polypropylene tubes at 37°C with frequent resuspensions to minimize adherence.

Discussion

These results indicate, for the first time, that flow cytofluorimetric analysis is a feasible and convenient method to analyze, at the level of individual cells, the procoagulant activity present on the membrane of monocytes. The method described here uses low cost materials and the signal produced by the fibrin needles made fluorescent by including fibrinogen-FITC in the reaction was strong and easy to quantify. The monocytes with PCA form fluorescent *sea urchin*-like cells which have 20-40 micron long needles of fibrin around the cell membrane and are localized as a distinct characteristic cloud in the side scatter versus green fluorescence diagram of the cytofluorogram; these properties made reliable discrimination of as few as 0.1% of positive cells possible. Although the appropriate concentration of heparin added to the developer plasma was critical to obtain well-shaped *sea urchin* cells it was easy to understand when the concentration was insufficient (leading to diffuse coagulation or to the formation of clumps of *sea urchin* cells with long fibers of fibrin in 10-15 min) or excessive (absence of *sea urchin* cells or absence of diffuse coagulation after 60 min). Some monocytes can initiate the coagulation on the cell surface but an appropriate amount of heparin is useful to further prolong the local polymerization of fibrin without leading to excessive propagation. A similar phenomenon may also occur *in vivo*: monocytes or macrophages surrounded by needles of fibrin are frequently observed when analyzing the effusion obtained from arthrocentesis in inflammatory arthritis, that is, in a place where the high content of chondroitin sulfate probably acts as a heparin-like anticoagulant. Observations regarding the capacity of monocytes and peritoneal macrophages to form *needles* or *stars* of fibrin date back to the 1970-80s, in studies initiating knowledge about the link between these cells and the clotting system.[10,11] In such studies it was clear that the polymerization of fibrin was due to some *hyperactive* monocytes but quantitative studies of these cells were not done. Thereafter, most studies on the PCA of monocytes were carried out by analyzing the capacity of a whole population of cells to accelerate the coagulation of recalcified plasma.

Recently, with the availability of monoclonal antibodies against tissue factor, several studies have used such antibodies to analyze cells: in suspension by cytofluorimetry, fixed cells in tissues by immunocytochemistry and soluble TF antigen by a sandwich ELISA assay.[12,13]

In our hands, flow cytometric analysis of TF antigen was complicated because the ability to quantify TF expression was impaired by the high level of non-specific fluorescence due to Fc receptors on

monocytes and by the absence of a distinct fluorescent positive population easily separable from the negative population after stimulation (i.e., continuous fluorescence from *negative* to *positive* regions). To quantify the percentage of monocytes with TF antigen accurately it was always necessary to perform control tests with isotype antibodies (*data not shown*).

Moreover, the quantity of TF antigen available for antibodies may not represent the real procoagulant activity because: a) as described in HL-60, a myeloid cell line, the inactive TF dimers present on the membrane may turn into active TF monomers after Ca^{++} influx into the cytosol following the addition of Ca^{++} ionophores, resulting in increased expression of binding sites for FVIIa and PCA;[14] b) the tissue factor pathway inhibitor (TFPI) protein is synthesized by several cell lines and cell types including stimulated monocytes, macrophages, vascular endothelium and is equally present in plasma and platelets. TFPI neutralizes TF procoagulant activity by binding to the factor Xa/VIIa/TF complex, leaving the TF epitope free to interact with the antibody.[14] Moreover, elastase, an enzyme widely present in neutrophils, can specifically inactivate TFPI and favor procoagulant activity;[15] c) many lipids, such as phosphatidylserine and phosphatidylcholine, can directly or indirectly modulate the membrane distribution of anionic phospholipids which are key co-factors for the surface assembly of procoagulant complexes and are essential for the acceleration of the TF-dependent initiation of blood coagulation.[16,17] It was also reported that PAF derived from activated platelets causes a rapid increase in activity of prior expressed tissue factor on monocytes.[18,19] But other lipids such as sphingosine can inhibit TF-FVIIa activity.[20] The lack of correlation between TF antigen and TF activity in monocytoid cells was described in a previous study.[21]

On the other hand, additional procoagulant activity on monocytes could be derived from a recently described TF-independent mechanism found in studies on monocytes[22,23] and fibroblasts, glial, and neuronal cells[24] in which the prothrombinase complex Xa/Va assembled on the membrane, favored by a specific membrane protein or by integrin CD11b, can lead to coagulation. Another study claimed that monocytes can initiate coagulation in a sequential three-step cascade, including binding of factor X to CD11b, discharge of azurophil granules after activation, and limited proteolytic activation of membrane-bound factor X by cathepsin G.[25,26]

The PCA of monocytes has implications in several pathologic conditions: in infections, elevated plasma tissue factor in patients with trauma and sepsis gives rise to thrombin generation, followed by intravascular coagulation.[27,28] Monocyte-derived circulating TF-containing microparticles seem to be the cause of DIC in meningococcal sepsis[29] and the risk of cerebral thrombo-embolism in bacteremic patients is very much higher than the corresponding risk in the general population.[30] In inflammation, it is commonly accepted that a period of hypercoagulability after trauma or surgery is due to a TF increase in monocytes.[31-33] In oncology, a procoagulant condition is frequent and enhanced TF expression in monocytes without stimulation or after LPS stimulation has been describe.[34,35] In atherogenesis, the monocyte and LDL recruiment to the intima initiates with increased adherence of monocytes to the arterial endothelium, adhesive reactions and interaction with activated platelets exposing P-selectin that results in increased TF expression and PCA in atherosclerotic plaques.[36] In diabetes, microangiopathy is related to an increased expression of tissue factor on monocytes.[37] In immunology, the PCA of monocytes and macrophages has long been known to be a result of cytokines produced mainly during alloantigenic recognition. Notably, the allogeneic procoagulant response was quantitatively discordant with respect to the allogeneic proliferative response, suggesting differences in specificity. This relatively rapid response may be applicable to typing of determinants in the major histocompatibility complex that are not equivalently identified by alternative analyses, and may be significant in tissue transplantation.[38] It was recognized that cytokines involved in cell-mediated immunity were responsible for the PCA response[39] and more specifically: the cellular response of the T CD4 lymphocytes with the Th1 reaction was responsible, the Th0 reaction to a lesser extent and the Th2 reaction was ineffective.[40] However, also antibodies such as anti PF4/heparin in heparin-induced thrombocytopenia and anti-cardiolipin in antiphospholipid syndrome bind to monocytes, leading to generation of PCA and then to thrombosis.[41,42] In most of the above pathologic conditions, a positive correlation was found between the quantity of PCA detected and the degree of clinical involvement.

Our data demonstrate that the number of monocytes with PCA forming fluorescent fibrin is roughly from a fifth to a third of the number of monocytes bearing TF antigen. This finding could indicate that only a fraction of monocytes with TF antigen are functionally important while the remainding cells are inactive or have a level of PCA insufficient to form a *sea urchin* of fibrin. In fact, prolonging the *developing* time beyond 10 min with an insufficient quantity of heparin did not increase the percentage of monocytes surrounded by fibrin but, rather, led to diffuse coagulation. Considering that the TF protein can interact with other molecules and modulate their function, it is possible that the method described here detects a minor but functionally active subpopulation of monocytes from all of those bearing tissue factor antigen. Further investigations

will clarify in which pathologic conditions this method of detecting monocytes with PCA could be most useful in helping diagnosis and guiding treatment. Moreover, the low number of monocytes required makes this method useful for *in vitro* studies examining many substances in different conditions that can influence the expression of procoagulant activity.

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Contributions

HR was responsible for the conception and design of the investigation. HR and GRM performed the laboratory experiments, analysis and interpretation. HR and GRM wrote the manuscript. HR is responsible for the table and figures.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Vicente Vicente, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Vicente and the Editors. Manuscript received July 23, 2002; accepted November 13, 2002. In the following paragraphs, professor Vicente summarizes the peer-review process and its outcomes.

What is already known on this topic

The procoagulant activity (PCA) of monocytes plays an important role in several physiologic and clinical conditions. However, methodology to analyze the procoagulant activity of monocytes seems to be complex.

What this study adds

This paper describes that flow cytofluorimetric analysis is a feasible and convenient method to investigate the procoagulant activity present on the membrane of monocytes.